

Engineering microbes to sense and eradicate *Pseudomonas aeruginosa*, a human pathogen

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

21 December 2010

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the two referees whom we asked to evaluate your manuscript. As you will see from the reports below, the referees raise substantial concerns on your work, which, I am afraid to say, preclude its publication.

The referees acknowledged that your work describes a functional synthetic circuit that can program *E. coli* to kill *P. aeruginosa* under laboratory conditions, however, they had substantial concerns regarding the therapeutic relevance of this technology. Both reviewers felt that it was not clear whether the culture results can be generalized to a disease-relevant state, with specific concerns regarding the density of *P. aeruginosa* needed to activate the *E. coli*, the outcomes of long-term culture (including resistance development), and the effects of biofilm formation. Reviewer #1 indicated clearly that, given these concerns and his/her feeling that the conceptual advance demonstrated by the synthetic genetic system was somewhat modest, they could not support publication of this work in Molecular Systems Biology.

The reviewers also felt that the system required additional characterization, in part to clearly demonstrate that both the lysis and the toxin modules are strictly required for *P. aeruginosa* killing. Both reviewers suggest potentially helpful experiments in this regard (e.g. testing on pyocin-resistant *P. aeruginosa*, and mutation of the lysis protein).

For the reasons stated above, we feel we have no choice but to return this manuscript with the message that we cannot offer to publish it.

Nevertheless, given the more encouraging comments from the second reviewer, and the possibility that these concerns could potentially be addressed with additional experiments, we may be willing to reconsider a new, substantially expanded, submission based on this work. Such a work would need

to provide additional, convincing experimental evidence supporting the therapeutic relevance of this approach, as well as better characterization of this system. We recognize that this would likely involve considerable further experimentation and analysis, and we would understand if, instead, you decide to submit this work to another journal. A resubmission would have a new number and receipt date, and we can give no guarantee about its eventual acceptability. However, if you do decide to follow this course then it would be helpful to enclose with your resubmission an account of how the work has been altered in response to the points raised in the present review.

I am sorry that the review of your work did not result in a more favorable outcome on this occasion, but I hope that you will not be discouraged from sending your work to Molecular Systems Biology in the future.

Thank you for the opportunity to examine this work.

Yours sincerely,

Editor
Molecular Systems Biology

Reviewer #1 (Remarks to the Author):

The paper by Saeidi et al proposes and explores a synthetic circuit for *E. coli* aimed at combatting *P. aeruginosa* infections. The core notion is to have both lysis and production of an anti-PA toxin (pyocin) expressed under a promoter that is triggered by quorum sensing autoinducers, expectedly produced by the very target PA cells. Authors show that the construct does work as expected and it seems to kill PA cells under Laboratory conditions. This is used as the basis to propose circuits of this sort as a therapeutic tool for dealing with pathogens, an alternative to antibiotics.

1. The ms. narrative abuses the jargon and discourse of Synthetic Biology. In contrast, the Abstract gives very little idea of what the paper is about. Half of the Introduction is just sheer advertisement: no reference to alternative anti-PA strategies.

2. The core construct(s) are trivial and predictable. If one places a lysis gene and a toxin gene behind and inducible promoter, cells will lyse and will release the toxin upon induction, no wonder. And if the inducer is itself produced by a toxin-sensitive strain, PA cell might be killed. This resembles a typical prey-predator game: the system may evolve either towards alternating oscillatory changes in the two populations (PA lyses EC and EC kills PA), or into a co-existence of mutants that either fail to produce pyocin (*E. coli*), or are resistant to it (PA), or both.

3. Despite the emphasis on the anti-PA value of the proposed scheme, Authors just base their more important claim in the very meagre data of Fig. 5. A control PA strain immune to pyocin is missing. There is no CFU count of either population present in the mixed culture(s). What is the meaning of the "relative fluorescence" of Fig5C? There is no indication of the minimal ratios EC/PA which are necessary to see an effect. One may require a dense population of PA to produce enough HSL. The efficiency of *E. coli* lysis in the mixed cultures is not examined. The evolution of PA and EC survival over times longer than 180 min is important to examine

4. While the EC / PG interplay game presented in the paper is intriguing, the two bacteria hardly share natural niches, they never meet. PA infection sites (lung, burnts, ear) are not amenable for *E. coli*. Furthermore, PA develops resistance to pyocins and other bacteriocins at quite high rate.

Reviewer #2 (Remarks to the Author):

Saeidi et al present a clever set of studies oriented towards creating *E. coli*-derived bacteria that exploit *Pseudomonas aeruginosa*'s use of quorum sensing to detect and then kill the pathogen. The obviously missing aspect of the manuscript is some demonstration of disease relevance in the form of a mouse study, or the demonstration that this works in the disease-relevant state for *Pseudomonas* (a biofilm) but the specific claims of the manuscript do not require that. On the whole, I find the overall idea a really good one, the studies generally well executed (with one exception below), and

the publication of high significance and interest. It's not the end of the story for this idea, but it's a key first-step publication. The manuscript is well written and readily-clear as to what they did and why.

I do have some question about whether the E7 device was correctly characterized. Other lysis systems (phage ones) cause complete disruption and the phenotype is self-evident from a drop in optical density, not just the halting of further growth. Growth rate and microscopy data is only evidence of a form of toxicity. What the authors should be showing is protein release. The literature-stated mechanism of E7 is some sort of lysis, but specifically what that means biochemically is not obvious to this reviewer-does it mean entire disruption of the integrity of the bacterium, disruption to the membranes, disruption of the peptidoglycan? In the context of its usage in the system described, the relevant question is whether or not it causes the release of protein. It isn't clear from the characterization whether the E7 device is releasing any proteins, periplasmic proteins, or all proteins. That could be assayed directly by showing a release of cytoplasmically-expressed or periplasmically-expressed reporter proteins which would be the relevant evidence to make such a claim of mechanism. Since the overall system is clearly working, and it seems reasonable to conclude that the pyocin is being released and that this is happening in response to AHL. By inference the E7 device might be working and causing the release of pyocin. However, that experiment would require that you also assay the cells with AHL directly regulating pyocin without the E7 and showing that this system doesn't work. As far as I can tell, that was not included. I would recommend that the authors quickchange a stop codon into the E7 and then do a killing assay. That would be minimally sufficient evidence of the stated mechanism.

Line92: misspelled *P. aeruginosa*

Line 93: I don't think I would describe a 498 amino acid protein as "small". That's medium-sized or larger-than-average.

Line 107: misspelled lactions

Resubmission

17 May 2011

Reply to the reviewers' comments

Comments by Reviewer 1

1. The narrative abuses the jargon and discourse of Synthetic Biology. Half of the Introduction is sheer advertisement. In contrast, the Abstract gives very little idea of what the paper is about.

The reviewer's suggestion has been incorporated into the manuscript. The text has been thoroughly revised for increased clarity.

2. No reference to alternative anti-PA strategies in Introduction.

The reviewer's suggestion has been incorporated into the manuscript. A brief literature review on alternative anti-PA strategies has been added to Introduction (lines 91 - 99).

*3. How *E. coli* and *P. aeruginosa* interplay in a mixed bacteria consortium, whether the system evolves towards alternating oscillatory changes, co-existence of mutants that fail to produce pyocin (*E. coli*) or are resistant to it (PA).*

We agree that the study of potential oscillatory behaviors of the *E. coli* and *P. aeruginosa* consortium could potentially be an interesting subject. However, the objective of this particular work was to demonstrate that our engineered *E. coli* senses and kills *P. aeruginosa* effectively in the mixed consortium.

4. Control PA strain immune to pyocin is missing. There is no CFU count of either population present in the mixed culture. There is no indication of the minimal ratio EC/PA to see an inhibition effect. There is concern that a dense population of PA might be required for sufficient HSL to

activate the E. coli system. The efficiency of E. coli lysis in final system is not examined. The evolution of PA and E. Coli survival over times longer than 180 min is important to examine.

The reviewer's suggestions have been incorporated into the study. New experiments were performed and the results were included in Results and Discussion. In summary, we showed that the engineered E. coli, carrying the final system, effectively inhibited the growth of PA in a 15-hour mixed culture when they were grown together at the EC/PA ratio of 4 (PA ~ 2×10^7 cfu/ml) (lines 285 - 288). We also validated (from CFU count and GFP measurement of green fluorescent PA cells) that the quorum sensing promoter in our engineered E. coli system was activated after PA began secreting homoserine lactones (HSL) in the late exponential phase. This corresponds to a PA cell density not exceeding 10^8 cfu/ml (Supplementary Figure 2C). We included a pyocin-resistant strain PAO1 (PA) which pyocin S5 was originally derived from in our inhibition studies, in both planktonic and biofilm co-cultures (lines 285 - 319). We also characterized the efficiency of the lysis device in the final system (i.e. pTetR-LasR-pLuxR-S5-pLuxR-E7) with HSL in terms of extracellular protein release and optical density measurement, using E. coli without the E7 lysis device as a control for comparison (i.e. pTetR-LasR-pLuxR-S5) (lines 233 - 245).

5. E. coli and PA do not share natural niches and never meet. The use of pyocin may promote the evolution of PA mutant strain resistant to pyocin.

The reviewer's concerns were addressed. Based on literature review, the acquisition of pyocin resistance in P. aeruginosa by horizontal gene transfer has not yet been discovered to date (lines 106 - 109). In addition, both E. coli and P. aeruginosa share common ecological niches along the gastrointestinal tract (lines 85- 87). Non pathogenic E. coli was used as the chassis in this study as a proof of concept. In future work, our synthetic biological system could be transferred to a probiotic chassis, for instance E. coli Nissle, to control Pseudomonas colonization of the GI tract. Health-promoting bacteria present in the upper respiratory tract can also be functionalized with our final system as novel bioagents to arrest Pseudomonas infection (lines 334 - 339).

Comments by Reviewer 2

1. Demonstration of disease relevance in the form of a mouse study or P. aeruginosa biofilm is missing.

The reviewer's suggestion was incorporated. New experiments were performed and the results were included in Results and Discussion. With the new results, we showed that the engineered E. coli, carrying the final system, effectively inhibited the growth of PA biofilm grown for 18 hours by CFU quantification of biofilm cells (lines 303 - 310) and observation under confocal laser scanning microscopy. (lines 311 - 319)

2. E7 lysis devices in both the standalone lysis system (i.e. pTetR-LasR-pLuxR-E7) and final system (i.e. pTetR-LasR-pLuxR-S5-pLuxR-E7) were inadequately characterized. A drop in optical density of engineered E. coli is not observed. The mechanism and biochemistry of E7 lysis is not obvious. In the context of this work, it is not clear whether the E7 device is capable of causing protein release. Authors should also show that the engineered system doesn't work without the E7 device.

The reviewer's suggestion was incorporated. New experiments were performed and the results were included in Results and Discussion. We showed that incomplete E. coli system without E7 lysis device (i.e. pTetR-LasR-pLuxR-S5) did not work well, and that only the final system (i.e pTetR-LasR-pLuxR-S5-pLuxR-E7), completed with sensing, killing and lysing devices, are was capable of inhibiting P. aeruginosa growth in both the planktonic (lines 293 - 298) and sessile states (lines 306 - 310 & lines 313 - 316). A literature review on the biochemistry of E7 lysis protein is presented in the Introduction section (lines 138 - 143). We also characterized E7 lysis devices in both the standalone lysis system and final system for a period of 6 hours to show a drop in optical density (lines 215 - 220). In addition, E7 lysis device in the final system was also characterized for the release of pyocin S5 using E. coli without the E7 lysis device as a control for comparison (i.e. pTetR-LasR- pLuxR-S5) (lines 233 - 245).

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the two referees who agreed to evaluate this revised study. As you will see, the referees opinions remained divided. The second reviewer was completely satisfied with the revisions made to this work and is now supportive of publication, while the first reviewer was less positive. For this reviewer, the most important concern appears to be the current lack of direct evidence supporting the applicability of this synthetic biology device in a disease-relevant system.

Given this divided opinion we sought advice from a member of our Editorial Board. This board member stated that "my strong opinion is that it is important to get this work out in the public domain," and indicated that requiring a direct proof of concept in a disease model would not be a reasonable standard in this case.

Given this clear advice, and the positive evaluation from the second reviewer, we feel that this work may be appropriate for publication after some minor modification.

1. In light of the first reviewer's concerns regarding the final applicability of this device in a disease setting, and we encourage you to use cautious language regarding the potential clinical relevance of this system, and to spend a few sentences in the Discussion openly acknowledging the need for future direct experimental testing in a disease-relevant model.

2. Molecular Systems Biology strongly encourages authors to provide the "source" numeric data associated with figure panels presenting quantitative analyses. To this end, we provide a new functionality that allows readers to directly download source data associated with selected figure panels (e.g. <<http://tinyurl.com/365zpej>>). The editor feels that this would be particularly appropriate for the data presented in this work. Please see out Instructions for Authors for preparation and submission guidelines (<<http://www.nature.com/msb/authors/index.html#a3.4.3>>).

3. Each Figure legend, when necessary, should describe the number of independent biological replicates represented in graphs, and what the error bars represent (s.e.m., standard deviation, etc.).

3. Please provide a single Supplementary Information pdf, including the supplementary figures and tables, with legends immediately below, and with a Table of Contents on the first page.

Please resubmit your revised manuscript online, with a covering letter listing amendments and responses to each point raised by the referees. Please resubmit the paper ****within one month**** and ideally as soon as possible. If we do not receive the revised manuscript within this time period, the file might be closed and any subsequent resubmission would be treated as a new manuscript. Please use the Manuscript Number (above) in all correspondence.

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Thank you for submitting this paper to Molecular Systems Biology.

Yours sincerely,

Editor - Molecular Systems Biology

Referee reports:

Reviewer #1 (Remarks to the Author):

I reviewed an earlier version of this ms and I raised a number of problems. Unfortunately, none of the critical points has been satisfactorily addressed in this new version. I was dismayed that, in their responses to reviewers, Authors mostly beat around the bush instead of producing unequivocal experiments in support of their claims. The work is not devoid of interest, but the claims (let alone the much insisted applicability of the proposed strategy) are not substantiated enough for making the story adequate for MSB.

Reviewer #2 (Remarks to the Author):

The authors have addressed the questions I raised during review.

1st Revision - authors' response

28 June 2011

Attached please find a revised manuscript entitled "Engineering microbes to sense and eradicate *Pseudomonas aeruginosa*, a human pathogen" (MSB-11-2953R) for re-review and publication in Molecular Systems Biology. In your previous review, several technical items were identified and they have now been corrected (please see item-by-item response). In particular, we have thoroughly revised the manuscript to ensure that the potential applicability of our engineered system is not overtly idealized and to acknowledge the need for further and more extensive experimentation in a direct disease setting, such as testing the system in vivo in a murine model. Further, we have provided source numeric data associated with figure panels presenting the quantitative analyses. We hope your evaluation finds the current version more in line with the scope of Molecular Systems Biology.

Reply to the Editor's comments

1. Suggestion to use cautious language regarding the potential clinical relevance of this system and to openly acknowledge the need for future direct experimental testing in a disease- relevant model.

Changes have been made in accordance to the editor's suggestion. The manuscript has been revised to ensure that the potential applicability of the current system is not overtly idealized and to acknowledge the need for further and more extensive experimentation in a direct disease setting, such as testing the system in vivo in a murine model. (Pg. 15, line 317 - 320)

2. To provide source numeric data associated with figure panels presenting the quantitative analyses.

The editor's suggestion has been implemented. We have standardized our source data in excel files accompanying the main manuscript, complying with the guidelines of MSB submission instructions.

3. To describe the number of independent replicates represented in graphs and what the error bars represent in each figure legends.

Changes have been made in accordance to the editor's suggestion. All figure captions have been revised to include the number of replicates and the representation of each error bar. (pg. 28 - 31, lines 642 - 722)

4. To provide a single Supplementary Information pdf, including the supplementary figures and tables, with legends immediately below and with a Table of Contents on the first page.

The editor's suggestion has been implemented. A supplementary information pdf, completed with a table of contents on the first page, is provided.